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Original Article

# Study of forced degradation behavior of pramlintide acetate by HPLC and LC–MS

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ABSTRACT

Pramlintide acetate (Symlin®), a synthetic analogue of the human hormone amylin. It was approved in March 2005 as a subcutaneous injection for the adjunctive treatment of patients who have type 1 or 2 diabetes mellitus. The objective of current investigation was to study the degradation behavior of pramlintide acetate under different ICH recommended stress conditions by HPLC and LC–MS. Pramlintide acetate was subjected to stress conditions of hydrolysis (acidic or alkaline), oxidation, photolysis and thermal decomposition. Extensive degradation products were observed under the hydrolysis, oxidation or thermal stress conditions, while minimal degradation was found in the photolytic conditions. Successful separation of drug from the degradation products was achieved by the validated chromatography (RP-HPLC and SCX-HPLC) methods. Subsequent to isolation, the molecular weight of each component was determined by LC–MS. The LC–MS *m/z* values and fragmentation patterns of 4 impurities matched with the predicted degradation products of pramlintide acetate.



## 1. Introduction

Pramlintide, a synthetic analogue of human hormone amylin with three prolines substitutions at position of 25, 28 and 29 [1], was approved in 2005 for adult use in patients with type 1 or type 2 diabetes mellitus and have failed to achieve glycemic control despite optimal therapy with insulin [2–4]. Like insulin, amylin is deficient in individuals with diabetes [5]. By mimicking endogenous amylin, pramlintide help the absorption of glucose by slowing the empty of gastric, promoting satiety via hypothalamic receptors, and inhibiting inappropriate secretion of glucagon, a catabolic hormone that opposes the effects of insulin and amylin [6–8].

The pramlintide peptide contains 37 amino acids with structure as below [9]:

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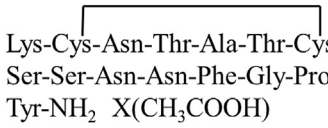


The amide at the C-terminal and the disulfide bond at 2 and 7 positions are crucial for retaining the full biological activity [7]. The 8 potential deamination sites ([Asn3, 14, 21, 22, 31, 35], [Gln10] and C-terminal amide) make some potentials for many deamination products that differ from pramlintide by modifications at only a single amino acid [10]. Moreover, hydrolysis, photolysis, oxidation and thermal stress are also potential causes of degradation for pramlintide peptide. Drug degradation may induce the safety and efficacy problems for patients through its unknown toxicity and lower the real uptake dose than expected [11] [12]. Information about the degradation products may improve pharmaceutical safety [13]. Therefore it is essential to study the stability of pramlintide acetate under various conditions, to identify and characterize the degradation products with combined analytical methods. Although the isolation and identification of heat stressed degradation products of pramlintide peptide was reported [10], the identification and confirmation of degradation products under other stress conditions was still unknown. In the present work, we investigated the degradation behavior of pramlintide acetate under different ICH recommended stress conditions such as hydrolysis (acidic or alkaline), oxidation, photolysis and thermal decomposition. Two HPLC methods were established to detect the degradation products of stressed pramlintide and the developed methods were validated to be effective to separate the drug and degradation products in each stressed condition. Pramlintide was found to be extensively unstable in alkaline, acidic, oxidation and thermal conditions but was relatively stable to photolytic stress. In additional, LC–MS was used to analyze the degradation products, the results indicated that the pramlintide acetate mainly degraded to [des-Pro$^{28}$]-PLLT, [des-Thr$^{4}$]-PLLT and [des-pro$^{25}$]-PLLT under such stressed conditions.

## 2. Experimental

### 2.1. Materials and reagents

#### 2.1.1. Chemicals

Acetonitrile (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany), pure water was purchased from Wahaha (Hangzhou, China). Other reagents which used in this study were of AR grade.

#### 2.1.2. Pramlintide acetate sample

Pramlintide acetate samples were quantitatively weighed and diluted to 0.5 mg mL$^{-1}$ in 30 mM acetate buffer (pH 4.0).

#### 2.1.3. Impurities mixed reference

Impurities No. 1 to 13 were quantitatively weighed and diluted to pramlintide acetate solution to make a mixture containing 0.05 mg mL$^{-1}$ of each impurities.

#### 2.1.4. Apparatus and chromatographic conditions

The HPLC system (Dionex) consisted of a P680 HPLC Pump, a UVD340U PDA detector and ASI-100 Automated Sample Injector and thermostatted Column Compartment Tcc-100. The data acquisition was performed by Chromeleon software version 3.1 (Dionex, Thermo Fisher Scientific Inc.), Waters Acquity UPLC-Waters Quattro premier XE, Column: Welch Ultimate Lp C8 (4.6 × 250 mm, 5 μm).

### 2.2. Stressed degradation studies

Stress degradation studies of pramlintide acetate were carried out under hydrolysis (acid and base), oxidation, photolytic and thermal conditions per ICH guidelines. Acidic hydrolysis of pramlintide acetate was carried out in 0.1 M HCl for 1 h and basic hydrolysis in 0.1 M NaOH for 7 min at room temperature respectively. For both hydrolysis conditions, each sample was neutralized with alkali or acid before dilution. Oxidative degradation of pramlintide acetate sample was carried out with 30% $H_2O_2$ at room temperature for 3 h. Photolytic studies were done by exposing a thin layer of the sample to day light for 5 days, a parallel set was kept in dark room as control. Pramlintide acetate was placed into penicillin bottle with cap and stamped with aluminum cover, kept at 105 °C in the oven for 3 h for thermal degradation study. All the solutions were filtered using 0.22 μm membrane filters before HPLC and LC–MS analysis.

### 2.3. Separation method

#### 2.3.1. Chromatographic condition

Two chromatographic conditions (RP-HPLC and SCX-HPLC) were optimized for separation of drug and degradation products in each stressed condition. The mixture of pramlintide acetate and 13 synthesized potential impurities were used to establish and optimize the chromatographic conditions for separating pramlintide acetate and the degradation products in two chromatographic conditions.

#### 2.3.2. Reversed-phase HPLC

The reversed-phase using a Waters® symmetry C8 column (250 mm × 4.6 mm, 5 μm particle size and 300 Å pore size). Column temperature was maintained at 45 °C. The flow rate was 0.3 mL min$^{-1}$ and detection wavelength was 215 nm. The mobile phase used was a mixture of 0.085 M monopotassium phosphate buffer (pH 3.0) and acetonitrile in a gradient program as shown in Table 4.

#### 2.3.3. Strong cation exchange (SCX) HPLC

The SCX column employed was a Polysulfoethyl-Aspartamide SCX column (100 mm × 4.6 mm, 5 μm particle size and 300 Å pore size, PolyLC®, USA). Column temperature was maintained at 40 °C. The flow rate was 0.8 mL min$^{-1}$ and detection wavelength was 215 nm. The separation achieved by a

mixture of buffer A (pH 5.8) and buffer B (PH 5.8) in a gradient program as shown in Table 5. The mobile phase buffer A was composed of 5 mM $KH_2PO_4$, 5 mM $NaClO_4$ and 40% $CH_3CN$, while mobile phase buffer B contained 5 mM $KH_2PO_4$,260 mM $NaClO_4$ and 40% $CH_3CN$.

### 2.4. LC–MS and MALDI-TOF/TOF

LC–MS studies were carried out on a system in which LC part was 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). The MS system was MicrOTOF-Q spectrometer (from Bruker Daltonik, Bremen, Germany). The whole system was operated using Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. Column temperature was maintained at 35 °C. The flow rate was 0.25 mL $min^{-1}$. The separation achieved by a mixture of TFA (Buffer A) and $CH_3CN$ (buffer B) in a gradient program as shown in Table 6. The mass spectrometer was run in negative ion mode with mass/charge (*m*/z) ratio in the range of 100–800 *m*/z, detection wavelength was 190–400 nm. MALDI-TOF/TOF system (5800 MALDI-TOF/TOF, AB SCIEX) was used to determine the molecular weight of four degradation products with detection range of 1000–8000 *m*/z. The MS data were analyzed by TOF/TOF Explorer and Data Explorer software.

## 3. Results and discussion

### 3.1. Development and optimization of isolation method

The initial literature collection showed that the heat stressed degradation products of pramlintide acetate were capable of successful isolation by using C8 reverse-phase column and Polysulfoethyl-Aspartamide SCX column [10,13]. So attempts were applied to develop a stability-indicating HPLC method on a C8 and SCX column. Serval modifications including change compositions of mobile phase and column temperature adjustment were tried to get good separations between the drug and the degradation products, as well as between the degradation products. The best separation was achieved on the C8 column at 45 °C using a mobile phase composed of 85 mM monopotassium phosphate buffer (pH 3.0) and acetonitrile in a gradient program as outlined in method.

With the condition described above, 20 μL of pramlintide acetate and a mixture of pramlintide acetate and 13 potential degradation products were analyzed to test the system suitability. The results showed that the solvent itself did not interfere with the determination of pramlintide and 13 potential products. The flat baseline of pramlintide acetate had been achieved. Twelve of the 13 potential products were well separated from the pramlintide by reversed-phase HPLC method (Fig. 1). The extent separation between two adjacent peaks was greater than 1.50, the number of theoretical plates and retention time all achieved the requirements as shown in Table 1.

Only [D-Lys$^1$]-PLLT (No. 9) was poorly separated from pramlintide acetate by RP-HPLC. Therefore, the SCX-HPLC gradient as specified in method was developed to resolve these two peaks (Fig. 2). The [D-Lys$^1$]-PLLT was successfully separated from the pramlintide acetate and the extent separation between two peaks, the number of theoretical plates and retention time all achieved the requirements as shown in Table 2.

### 3.2. Stress studies

With the condition which was described above, the SCX-HPLC and RP-HPLC methods that combined with LC–MS were used to analyze the pramlintide acetate samples which were respectively subjected to hydrolysis (acidic or alkaline), oxidation, photolysis or thermal stress conditions. The results suggested the degradation behavior as follow.

#### 3.2.1. Acidic condition

Acidic hydrolysis of pramlintide acetate was carried out in a solution of 0.1 M HCl for 1 h at room temperature. Pramlintide acetate was found to be labile in acidic condition. Degradation of the drug resulted in the rise of 16 additional products (Fig. 3A) comparing with pramlintide acetate control (Fig. 3F). As shown in Fig. 3A, three of the major peaks showed up at ~79 min (NO. 17, RRT = 1.07), ~85 min (NO. 18 RRT = 1.15) and ~91 min (NO. 19, RRT = 1.21), which were corresponded to the substance of [des-Pro$^{28}$]-PLLT (NO. 11), [des-Thr$^4$]-PLLT (NO. 12) and [Ac-Lys$^1$]-PLLT (NO. 13) in Fig. 1. The mass spectroscopy indicated these three degradation products had molecular weights of *m*/z 3850.63, 3946.69 and 3989.71, which matched with the predicted substance of [des-Pro$^{28}$]-PLLT, [des-Thr$^4$]-PLLT and [Ac-Lys$^1$]-PLLT (Table 3).

#### 3.2.2. Alkaline condition

Compared to the results of acidic condition, pramlintide acetate was found to be more sensitive to alkaline condition. In 0.1 M NaOH buffer, pramlintide acetate quickly degraded to more than 20 additional fragments within 7 min at room temperature compared with pramlintide acetate control. As shown in Fig. 3B, The HPLC chromatogram indicated that three of the degradation products NO. 20 (~79 min, RRT = 1.07), NO. 21 (~86 min, RRT = 1.15) and NO. 22 (~95 min, RRT = 1.27) in alkaline condition correspond to the predicted substance of [des-Pro$^{28}$]-PLLT (NO. 11), [des-Thr$^4$]-PLLT (NO. 12) and [des-pro$^{25}$]-PLLT (NO. 14) in Fig. 1. The *m*/z of these three degradation products were 3850.63, 3946.69 and 3892.87 which matched with the predicted substance of [des-Pro$^{28}$]-PLLT, [des-Thr$^4$]-PLLT and [des-pro$^{25}$]-PLLT (Table 3).

#### 3.2.3. Thermal degradation

Pramlintide acetate was placed into penicillin bottle with cap and stamped with aluminum cover, kept at 105 °C in the oven for 3 h. Similar degradation behavior was observed between samples exposed to thermal stress and alkaline stress. Pramlintide acetate quickly degraded to more than 20 fragments comparing with pramlintide acetate control, but there were no obviously major degradation product could be analyzed (Fig. 3C).

#### 3.2.4. Oxidative conditions

Oxidative degradation of pramlintide acetate sample was carried out by exposing to 30% $H_2O_2$ for 3 h at room

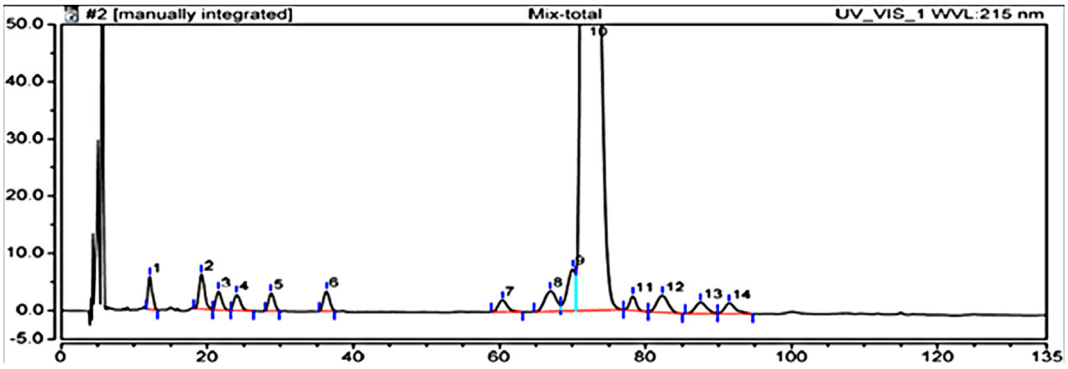


**Fig. 1 – Chromatogram of mixture of pramlintide acetate and 13 potential degradation products by Reversed-phase HPLC method.**

**Table 1 – Separation results of pramlintide acetate and potential degradation products by RP-HPLC method.**

| NO. | Substance | Retention time (min) | Rel Ret time (PLLT) | Plates (EP) | Resolution (EP) |
|---|---|---|---|---|---|
| 1 | [des-Ala$^{13}$]-PLLT | 12.108 | 0.17 | 2193 | 5.91 |
| 2 | [D-Ala$^{5}$]-PLLT | 19.217 | 0.27 | 3111 | 1.64 |
| 3 | [D-Phe$^{15}$]-PLLT | 21.535 | 0.30 | 3516 | 1.61 |
| 4 | [D-Phe$^{23}$]-PLLT | 24.033 | 0.33 | 3368 | 3.05 |
| 5 | [D-Ala$^{8}$]-PLLT | 28.748 | 0.40 | 6318 | 5.04 |
| 6 | [D-Ala$^{5}$]-PLLT | 36.298 | 0.51 | 8678 | 12.90 |
| 7 | [des-Ala$^{5}$]-PLLT | 60.418 | 0.84 | 12,152 | 2.52 |
| 8 | [D-His$^{18}$]-PLLT | 67.037 | 0.93 | 7617 | N/A |
| 9 | [D-Lys$^{1}$]-PLLT | 70.035 | 0.98 | 8927 | N/A |
| 11 | [des-Pro$^{28}$]-PLLT | 78.283 | 1.09 | 38,735 | 1.80 |
| 12 | [des-Thr$^{4}$]-PLLT | 82.302 | 1.15 | 12,920 | 1.86 |
| 13 | [Ac-Lys$^{1}$]-PLLT | 87.583 | 1.22 | 15,615 | 1.50 |
| 14 | [des-Pro$^{25}$]-PLLT | 91.503 | 1.27 | 22,160 | N/A |
| 10 | Pramlintide acetate | 71.820 | 1.00 | 8927 | 2.80 |

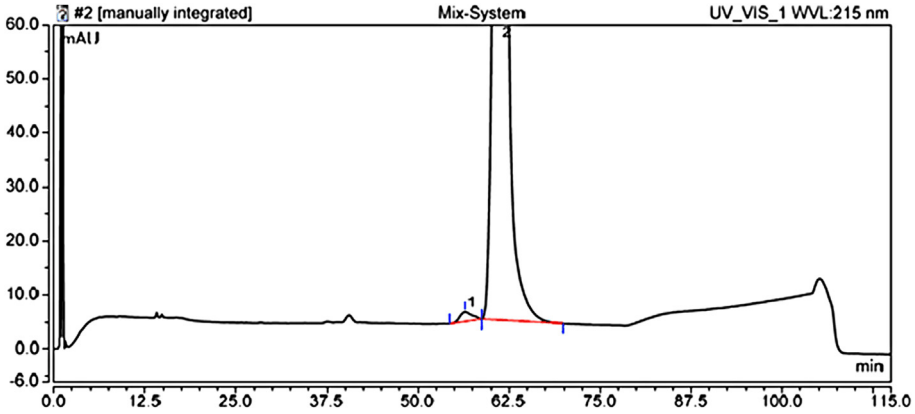


**Fig. 2 – Chromatogram showing separation of pramlintide acetate and potential degradation substance [D-Lys$^{1}$]-PLLT by SCX-HPLC method.**

**Table 2 – Separation results of pramlintide acetate and potential degradation products by SCX-HPLC method.**

| NO. | Substance | Retention time (min) | Rel Ret time (PLLT) | Plates (EP) | Resolution (EP) |
|---|---|---|---|---|---|
| 1 | [D-Lys$^{1}$]-PLLT | 12.108 | 0.17 | 2193 | 5.91 |
| 2 | Pramlintide acetate | 19.217 | 0.27 | 3111 | 1.64 |

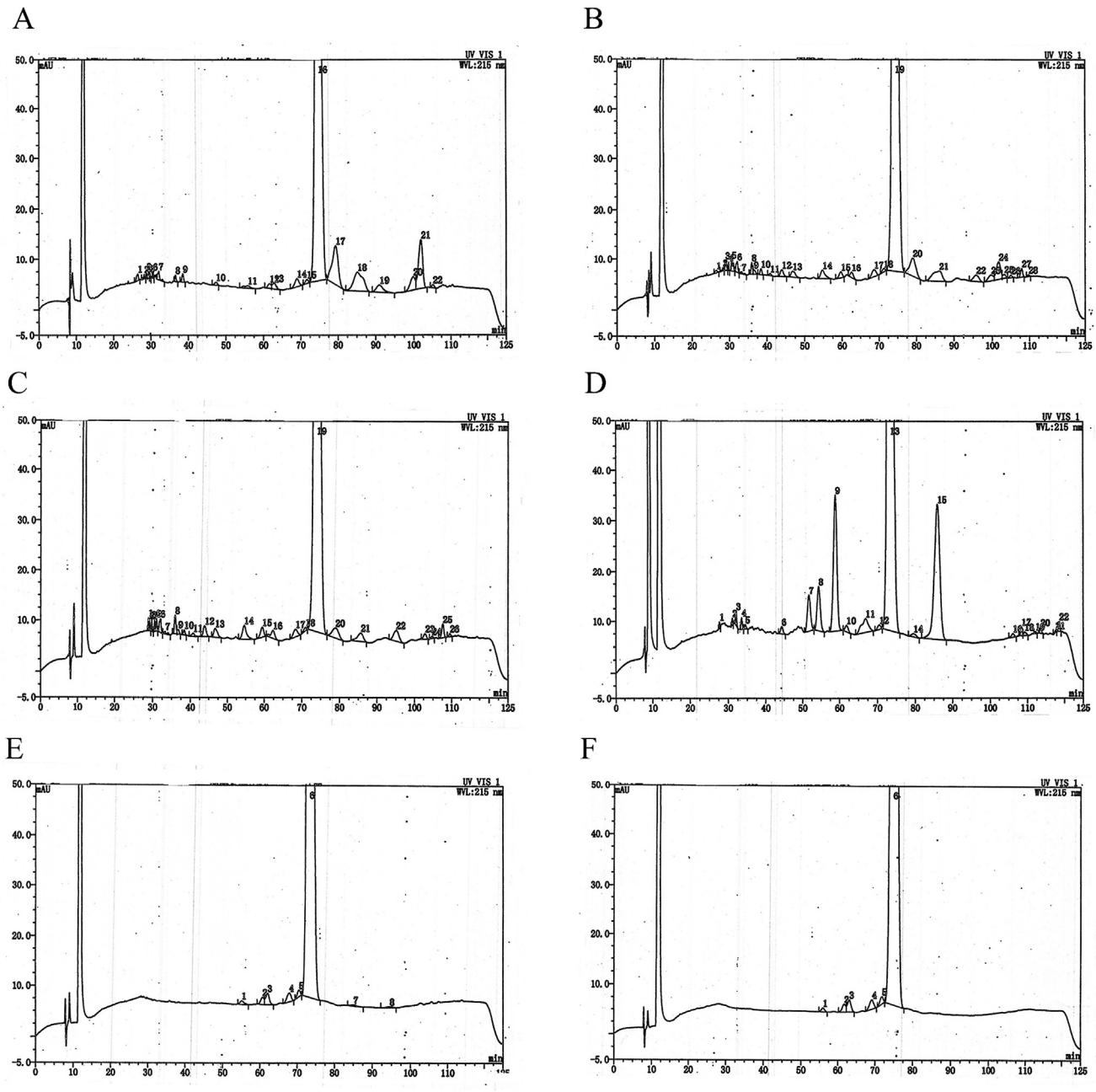


**Fig. 3 – Representative HPLC chromatograms of pramlintide acetate stressed sample. (A). Sample degraded in 0.1 M HCl at room temperature. (B). Sample degraded in 0.1 M NaOH. (C). Sample subjected to thermal degradation. (D). Sample subjected to oxidative degradation. (E). Sample subjected to photolytic degradation. (F). Chromatogram of pramlintide acetate control.**

temperature. Two major degradation peaks were observed in the chromatogram (Fig. 3D). The relative retention time of NO. 14 (~80 min, RRT = 1.09) and NO. 15 (~86 min, RRT = 1.16) of the degradation peaks were very similar to that of [des-Pro$^{28}$]-PLLT (NO. 11) and [des-Thr$^{4}$]-PLLT (NO. 12) in Fig. 1. LC–MS investigation confirmed the *m/z* of these two degradation products were 3850.63 and 3946.69, which showed that pramlintide acetate mainly degraded to [des-Pro$^{28}$]-PLLT and [des-Thr$^{4}$]-PLLT (Table 3).

### 3.2.5. *Photolytic conditions*

Photolytic studies were done by exposing a thin layer of the sample to day light for 5 days, a parallel control set was kept in dark room. Negligible degradation was observed on subjecting

**Table 3 – Summary of MALDI-TOF/TOF results of major degradation products.**

| Degradation product | Observed *m/z* | Proposed structure | Theoretical molecular weight |
|---|---|---|---|
| [des-Pro$^{28}$]-PLLT | 3853.63 | H-Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu<br>Pro-Gly-Phe-Asn-Asn-Ser-Ser-His-Val-Leu-Phe-Asn-Ala<br>Ile-Leu-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-$NH_2$ | 3852.25 |
| [des-Thr$^{4}$]-PLLT | 3849.69 | H-Lys-Cys-Asn-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu<br>Pro-Gly-Phe-Asn-Asn-Ser-Ser-His-Val-Leu-Phe-Asn-Ala<br>Ile-Leu-Pro-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-$NH_2$ | 3848.3 |
| [Ac-Lys$^{1}$]-PLLT | 3988.93 | Ac-Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu<br>Pro-Gly-Phe-Asn-Asn-Ser-Ser-His-Val-Leu-Phe-Asn-Ala<br>Ile-Leu-Pro-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-$NH_2$ | 3989.71 |
| [des-Pro$^{25}$]-PLLT | 3853.87 | H-Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu<br>Ile-Gly-Phe-Asn-Asn-Ser-Ser-His-Val-Leu-Phe-Asn-Ala<br>Leu-Pro-Pro-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-$NH_2$ | 3852.25 |

**Table 4 – Gradient program for finally developed RP-HPLC method.**

| Time (min) | Phosphate buffer (%) | Acetonitrile (%) |
|---|---|---|
| 0.0 | 88 | 12 |
| 1.0 | 88 | 12 |
| 16.0 | 72 | 28 |
| 85.0 | 72 | 28 |
| 100.0 | 68 | 32 |
| 110.0 | 68 | 32 |
| 110.5 | 88 | 12 |
| 135.0 | 88 | 12 |

**Table 5 – Gradient program for finally developed SCX-HPLC method.**

| Time (min) | Buffer A (%) | Buffer B (%) |
|---|---|---|
| 0.0 | 98 | 2 |
| 6.0 | 85 | 15 |
| 6.5 | 85 | 15 |
| 24.0 | 48 | 52 |
| 61.0 | 48 | 52 |
| 81.0 | 12 | 88 |
| 86.0 | 12 | 88 |
| 91.0 | 98 | 2 |
| 115.0 | 98 | 2 |

**Table 6 – Chromatographic conditions for LC–MS method.**

| Time (min) | Buffer A (%) | Buffer B (%) |
|---|---|---|
| 0 | 70 | 30 |
| 2 | 70 | 30 |
| 15 | 30 | 70 |

the drug to day light for 5 days compared with the pramlintide acetate control by HPLC (Fig. 3E).

## 4. Conclusion

In this study, pramlintide acetate was subjected to stress studies under various ICH recommended conditions. Two HPLC methods were established to detect the degradation products of stressed pramlintide and the developed methods were validated to be effective to separate the drug and degradation products in each stressed condition. Pramlintide was found to extensively degrade in alkaline, acidic, oxidation and thermal conditions, but was relatively stable to photolytic stress. In additional, LC–MS was used to analysis the degradation products, the results indicated that the pramlintide acetate mainly degraded to [des-Pro$^{28}$]-PLLT,

[des-Thr$^{4}$]-PLLT and [des-pro$^{25}$]-PLLT in the stressed conditions of this study.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## REFERENCES


[1] Colburn WA, Gottlieb AB, Koda J, Kolterman OG. Pharmacokinetics and pharmacodynamics of AC137 (25,28,29 tripro-amylin, human) after intravenous bolus and infusion doses in patients with insulin-dependent diabetes. J Clin Pharmacol 1996;36:13–24.

[2] Schmitz O, Brock B, Rungby J. Amylin agonists: a novel approach in the treatment of diabetes. Diabetes 2004;53:S233–8.

[3] Edelman S, Maier H, Wilhelm K. Pramlintide in the treatment of diabetes mellitus. BioDrugs 2008;22:375–86.

[4] Thompson RG, Pearson L, Schoenfeld SL, Kolterman OG. Pramlintide, a synthetic analog of human amylin, improves the metabolic profile of patients with type 2 diabetes using insulin. Diabetes Care 1998;21:987–93.

[5] Cooper GJ. Amylin and insulin co-replacement therapy for insulin-dependent (type I) diabetes mellitus. Med Hypotheses 1991;36:284–8.

[6] da Silva DC, Fontes GN, Erthal LC, Lima LM. Amyloidogenesis of the amylin analogue pramlintide. Biophys Chem 2016;219:1–8.

[7] Adeghate E, Kalász H. Amylin analogues in the treatment of diabetes mellitus: medicinal chemistry and structural basis of its function. Open Med Chem J 2011;5:78–81.

[8] Hay DL, Chen S, Lutz TA, Parkes DG, Roth JD. Amylin: pharmacology, physiology, and clinical potential. Pharm Rev 2015;67:564–600.

[9] Cort JR, Liu Z, Lee GM, Huggins KNL, Janes S, Prickett K, et al. Solution state structures of human pancreatic amylin and pramlintide. Protein Eng Des Sel 2009;22:497–513.

[10] Hekman C, DeMond W, Dixit T, Mauch S, Nuechterlein M, Stepanenko A, et al. Isolation and identification of peptide degradation products of heat stressed pramlintide injection drug product. Pharm Res 1998;15:650–9.

[11] Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs—a review. J Pharm Anal 2014;4:159–65.

[12] Chen WH, Lin YY, Chang Y, Chang KW, Hsia YC. Forced degradation behavior of epidepride and development of a stability-indicating method based on liquid chromatography–mass spectrometry. J Food Drug Anal 2014;22(2):248–56.

[13] Hekman CM, DeMond WS, Kelley PJ, Mauch SF, Williams JD. Isolation and identification of cyclic imide and deamidation products in heat stressed pramlintide injection drug product. J Pharm Biomed Anal 1999;20:763–72.